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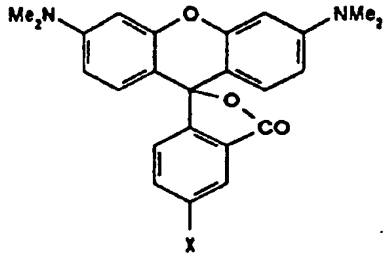
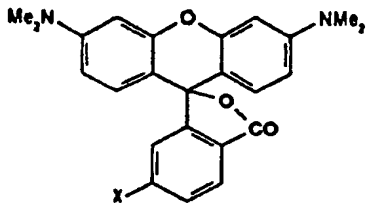
(54) Title: CHEMICAL SYNTHESIS OF RHODAMINE DERIVATIVES




(a)

(5 isomer)




(b)

(6 isomer)

(57) Abstract

A method for preparing rhodamine derivatives of formulae (a) or (b) or a mixture of such compounds, where X is bromo, chloro- or iodoacetamido, maleimido, or amino. These compounds are the 5 and 6 (or 5' and 6') isomers of the amine, haloacetamide and maleimide derivatives of tetramethylrhodamine. The method enables for the first time the production of isomerically pure compounds on a relatively large scale. The products are fluorescent and are useful for the labelling of proteins.

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# CHEMICAL SYNTHESIS OF RHODAMINE DERIVATIVES

This invention relates to a new process for the preparation of rhodamine derivatives; more particularly the 5 and 6 (or 5´ and 6´) isomers of the amine, haloacetamide and maleimide derivatives of tetramethylrhodamine. The process enables for the first time the production of isomerically pure compounds on a relatively large scale. The products are fluorescent and are useful for the labelling of proteins.

Rhodamines are well known as dyes or staining agents and as fluorescent labels, and are widely used in microscopy and other techniques for the study of the structure and dynamics of cellular and other biological systems. They are members of the triphenylmethane group, closely related to fluorescein, and have traditionally been prepared by the condensation of phthalic anhydride with N-alkylated m-aminophenols in the presence of concentrated sulphuric acid.

Iodoacetamidotetramethylrhodamine (IATR) is well known for use in the labelling of proteins, where it binds to the thiol groups of any cysteine sidechains present, and there are numerous literature references describing such uses. Among the earliest references are J. Borejdo et al., Proc. Natl. Acad. Sci. U.S.A., 1979, 76, 6346 and A. Levi et al., Proc. Natl. Acad. Sci. U.S.A., 1980, 77, 3469. The literature is almost exclusively concerned with work involving the use of IATR and there are no openly available reports on processes for its preparation.

One of the most widely used of the commercially available IATR preparations is in fact a

mixture of the 5 isomer and the 6 isomer. However, problems have been encountered in obtaining results which can be consistently and accurately reproduced. This is thought likely to be due to the proportions of the two isomers varying between different batches of the preparation, and perhaps also the presence of impurities which can obviously diminish the labelling performance. K. Ajtai et al. (Biochemistry, 1992, 31, 12431-12440) have reported an inability to reproduce earlier results when using such a mixed isomer IATR probe for labelling muscle fibre proteins. Further investigations using samples of purified isomers revealed that the preparation used in the first experiments was predominantly the 5 isomer, while the preparation used latterly contained mainly the 6 isomer. According to Ajtai et al. (op. cit.) while both of these isomers of IATR will label the muscle fibre proteins, they apparently do so at different rates and only the 5 isomer affects $K^+$-EDTA- and $Ca^{2+}$ - activated ATPases. Thus, differences in the ratios of the two isomers present in a labelling preparation can have a profound effect on the results obtained. To the best of our knowledge isomerically and chemically pure preparations of either the 5 isomer or the 6 isomer of IATR are not available commercially; only mixtures of the two isomers are sold.

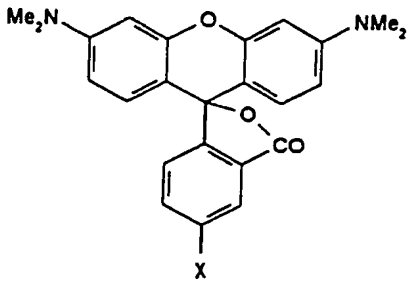
As previously noted, rhodamines are structurally related to fluoresceins and the latter compounds are also well known for use as fluorescent labels. The normal method of synthesis produces 5- and 6-substituted nitrofluoresceins, which can be separated by fractional crystallisation of the mixed diacetates (A.H. Coons et al., J. Exp. Med., 1950, 91, 1-13). H.S. Corey et al. (Nature, 1966, 212, 1040-1042) assigned structures to the 5-nitrofluorescein and

6-nitrofluorescein isomers using nuclear magnetic resonance spectroscopy (NMR) and these separate nitrofluoresceins can be reduced to aminofluoresceins, which serve as starting materials for elaboration into protein labelling reagents. However, processes for the preparation of rhodamines based on analogous chemistry have not proved to be particularly efficient.

S.D. Clarke (Ph.D. Thesis, Cambridge University 1990, p. 116) describes the use of ethyl polyphosphate (polyphosphoric ester or PPE) as a catalyst for the preparation of rhodamines. Anhydrous zinc chloride and other Lewis acids have also been employed in the past for this purpose (e.g. G.A. Smith et al., J. Chem. Soc., Perkin Trans. II, 1993, 1195). E.M. Berman et al. (J. Org. Chem., 1989, 54, 5642-5644) have described the use of trimethylsilyl polyphosphate (PPSE) to promote intramolecular cyclisations in Friedel-Crafts reactions leading to the preparation of 9H-selenoxanthen-9-ones. There is no teaching or suggestion, however, of its use as a catalyst in a process for the preparation of rhodamines and which involves intermolecular (rather than intramolecular) Friedel-Crafts reaction.

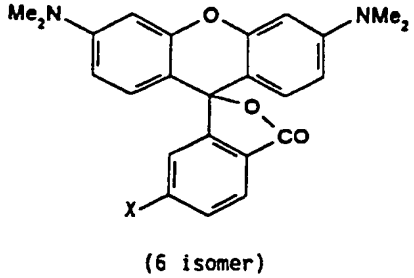
There is therefore a need for an improved process for synthesising rhodamines and, in particular, for a process which is straightforward and can be used for the direct and efficient production of compounds that are isomerically and chemically pure. The present invention seeks to provide such a process.

According to the present invention there is provided a method for preparing compounds of the following formulae:-



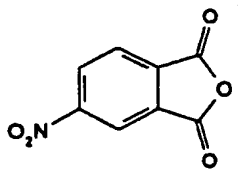
(5 isomer)

or



(6 isomer)

or a mixture of such compounds,
where X is bromo-, chloro- or iodo- acetamido,
maleimido, or
amino;
which comprises the following sequence of reactions:-
I)



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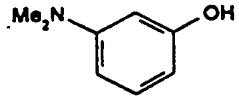
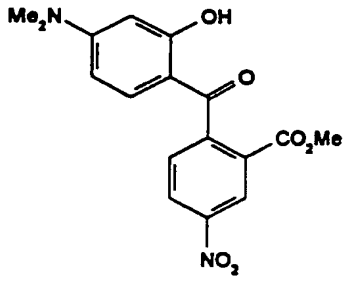
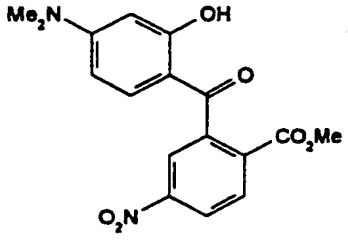


1) toluene/Δ
2) MeOH/H+



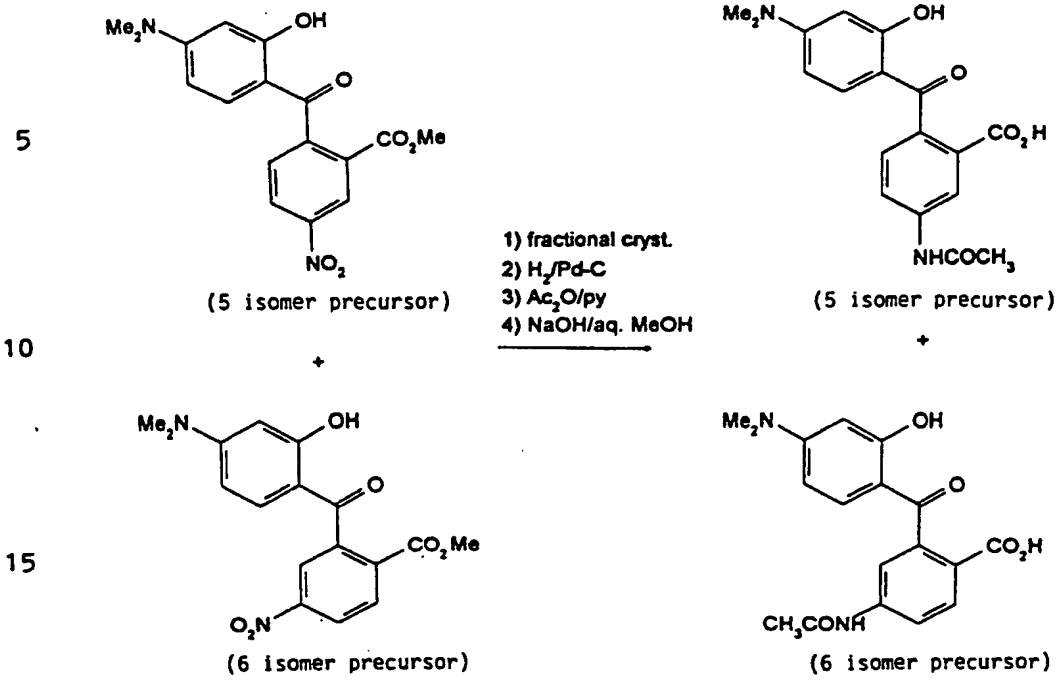
(5 isomer precursor)

+

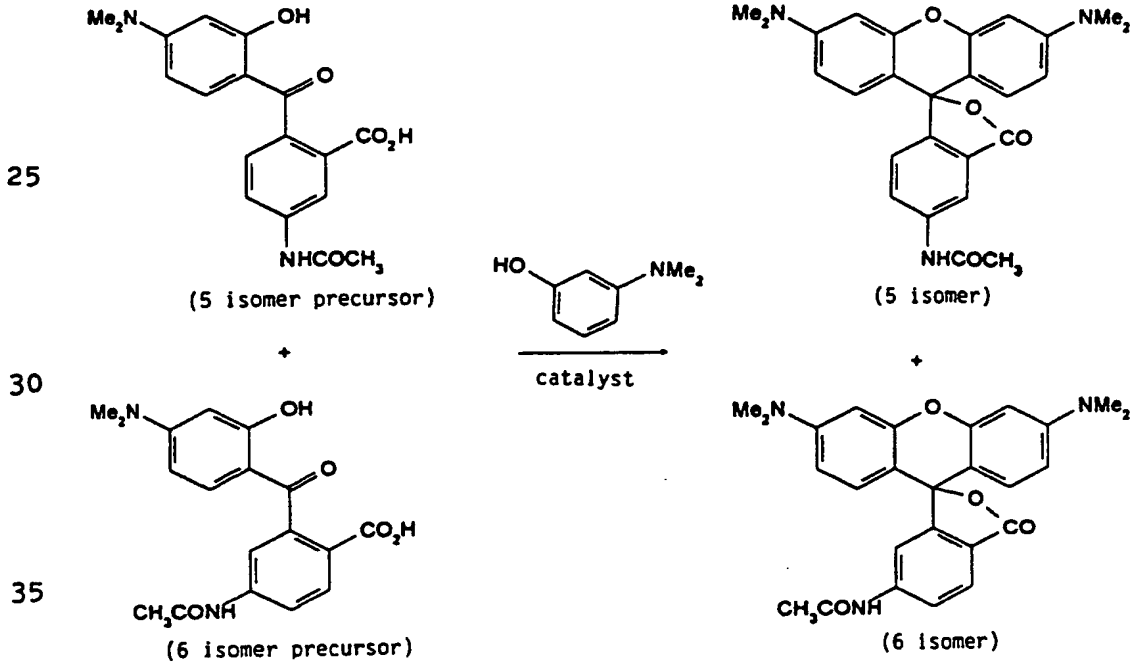


(6 isomer precursor)

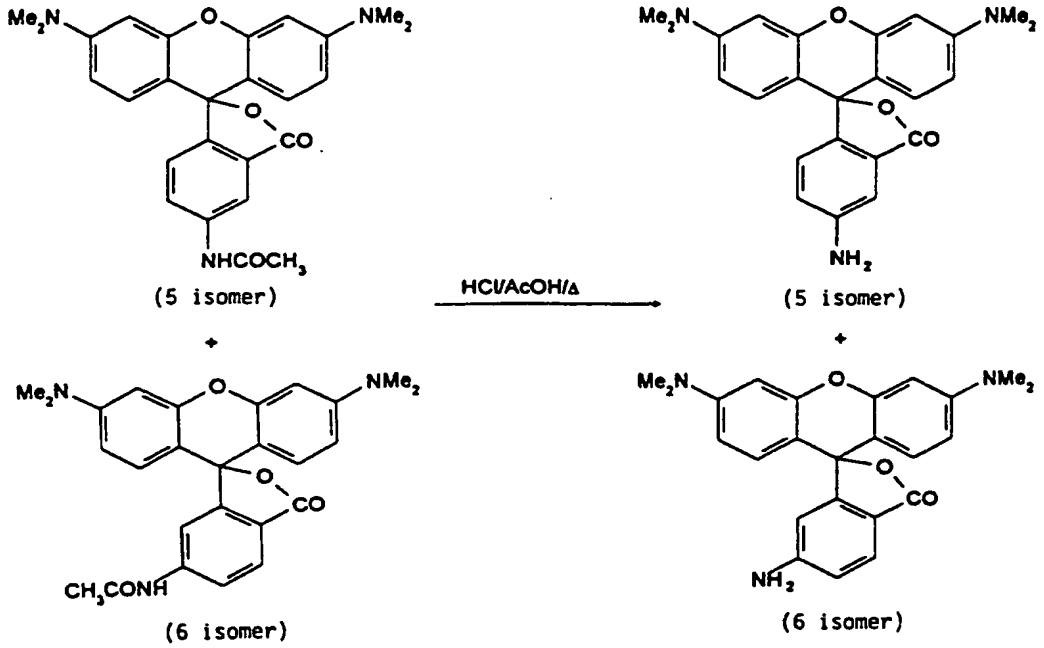
II) reduction of the nitro group:




III) formation of the rhodamine structure in the presence of a catalyst:

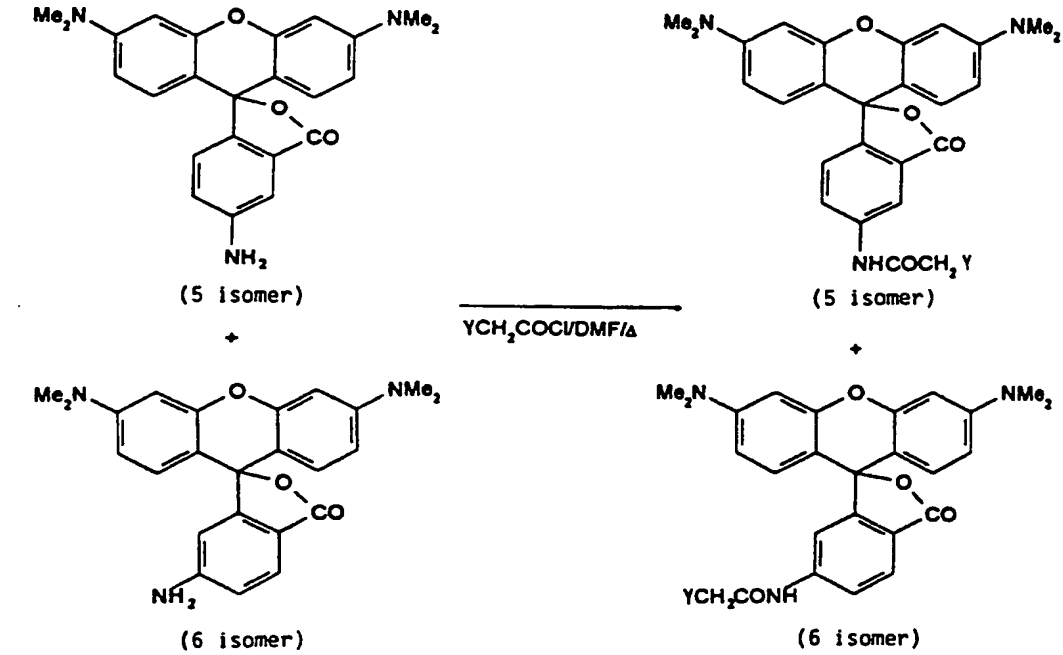


IV) formation of the amine derivative:




and then, optionally,

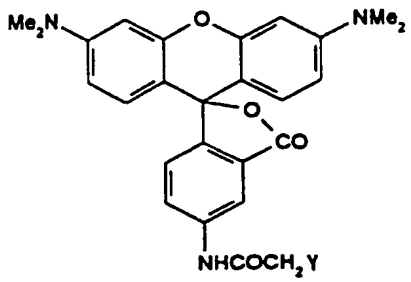
V) conversion of the amine derivative obtained in step IV) to the bromo- or chloro- acetamide derivative:




where Y is Br or Cl;

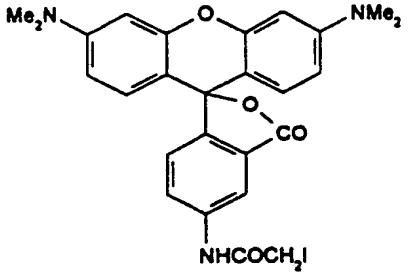
and then, optionally,

VI) conversion of the bromo- or chloro- acetamide derivative obtained in step V) to the iodoacetamide derivative:




(5 isomer)

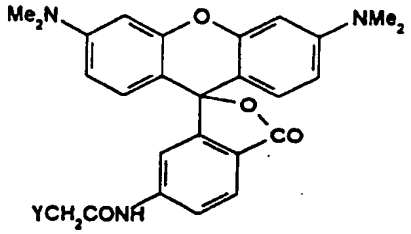
+




(5 isomer)

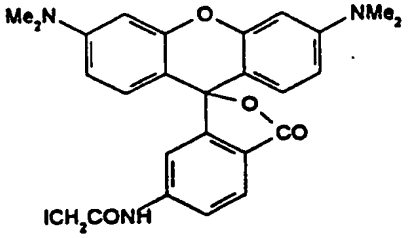
+

NaI/MeOH




(6 isomer)

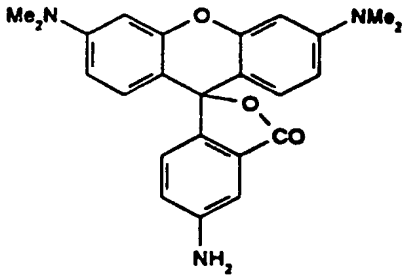
+



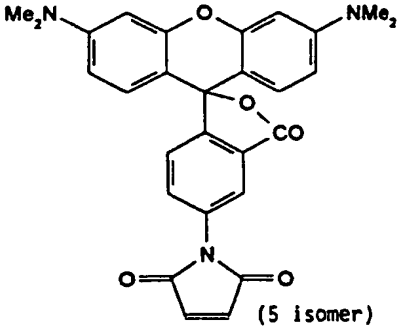

(6 isomer)

or, alternatively,

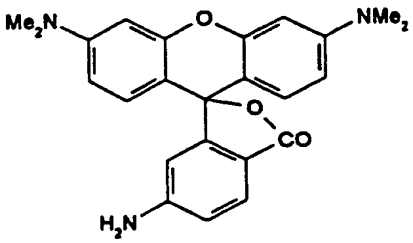
VII) conversion of the amine derivative obtained in step IV) to the maleimide derivative:

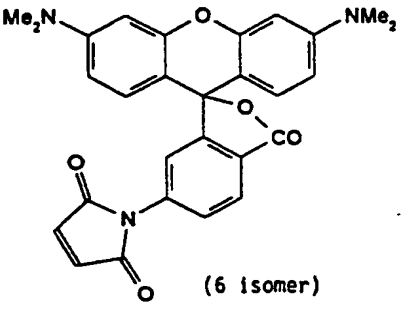



1) Maleic anhydride
2) $Ac_2O$/NaOAc/Δ



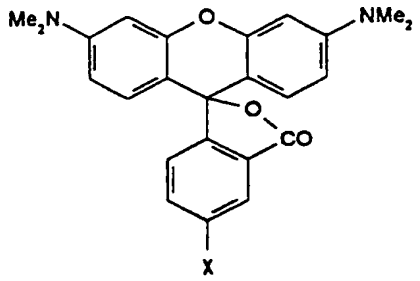

(5 isomer)




(6 isomer)

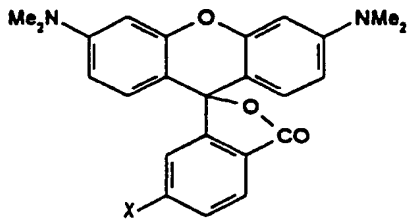
According to the present invention there is further provided a compound of the following formula:




(5 isomer)

where X is as defined above, in a substantially pure form. X is most preferably iodoacetamido (-$NHCOCH_2I$).

According to the present invention there is further provided a compound of the following formula:




(6 isomer)

where X is as defined above, in a substantially pure form. X is most preferably iodoacetamido (-$NHCOCH_2I$).

According to the present invention there is still further provided a method of investigating or determining protein orientation, structure or movement which comprises the use of a compound made by the aforementioned method of this invention.

The method of this invention can be used to

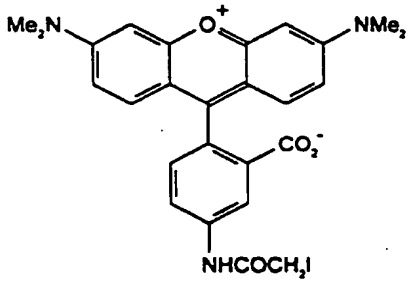
prepare either 5 isomer products and/or 6 isomer products separately, or a mixed isomer product containing both the 5 and the 6 isomers. If it is desired to prepare an isomerically pure product, the separation of the isomers from one another can be performed at the end of either step I) or step II) above - but is usually most conveniently done between step I) and step II). The separation is typically achieved by means of crystallisation procedures and the isomers can be identified and distinguished using conventional NMR procedures.

It will be appreciated that if the 5 isomer and the 6 isomer precursors are separated from one another at an early stage in the method, and the subsequent steps performed on either one of them or on each of them in isolation, isomerically pure products will be obtained. Alternatively, if the isomer precursors are not separated, the method can be used to produce a mixed isomer product containing both the 5 isomer and the 6 isomer. Furthermore, the method of this invention is a multi-step process. It will be appreciated that the method would not be carried out beyond step IV) if the amine derivative is the desired end-product. Stopping the method at step V) would produce the bromo- or chloro- acetamide derivative, while continuing to step VI) results in the iodoacetamide derivative. As an alternative to steps V) and VI), step VII) is performed if the maleimide derivative is the desired product.

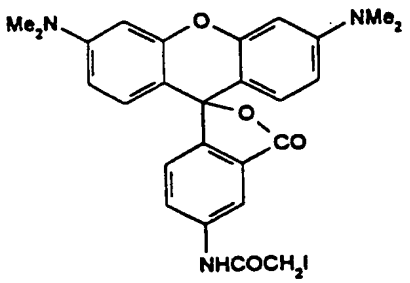
The method involves the use of a catalyst in step III) above. This could be any of the catalysts previously known for use with rhodamines, such as sulphuric acid, PPE, anhydrous zinc chloride and other Lewis acids. Most preferably, however, the catalyst employed is PPSE. It has been found that PPSE is

surprisingly effective in promoting the reaction and results in a much improved yield. For example, using sulphuric acid as the catalyst a yield of around 10% can typically be expected. With PPSE, the yield may be increased to as much as 70-80%.

It is well known that rhodamines can exist as either of two valence tautomeric forms: a fluorescent xanthylium form or a non-fluorescent spirolactone form. For example:-




Xanthylium form of 5 isomer




Spirolactone form of 5 isomer

It is to be understood that the method of this invention can be used to produce compounds in either of these forms. The fluorescent tautomer is of greater utility but for consistency with the conventional numbering system, the compounds are depicted in their spirolactone form only.

The present method produces rhodamines in high yield and also provides a convenient route for the production of isomerically pure compounds. Using the method, single isomer products with a purity of at least 95% (and generally at least 99%) have been obtained. Indeed, in some cases the final product has been found to be greater than 99.9% isomerically pure (i.e. contaminated with less than 0.1% of the other isomer).

The products of this invention (in particular the iodoacetamide and maleimide derivatives) can be used like conventionally produced rhodamines as dyes or stains and as fluorescent labels in both qualitative and quantitative experimental work and research studies. The availability for the first time of isomerically and chemically pure products with unambiguously defined structures can be expected to lead to labelled reagents (such as antibodies) of improved quality. This should in turn bring about an improvement in the accuracy and reproducibility of experimental results, particularly in quantitative work.

The method and compounds of this invention will now be further illustrated by the following Example. The numbering of the intermediates and end-products corresponds to that on the accompanying Reaction Sequence 1 and Reaction Sequences 2A and 2B.

**Experimental**

Melting points were determined on a Reichert hot stage microscope and are uncorrected. Analyses were carried out by the Chemical Analysis Centre, University of Kent, Canterbury. Mass spectra were determined on a VG 70-250SE instrument. NMR spectra were, unless otherwise stated, determined in $CDCl_3$ on JEOL FX90Q and Bruker WM400 spectrometers with tetramethylsilane as the internal standard; *J* values are given in Hz. The structures of the isomeric nitrobenzoates 7 and 8 were determined from their $^1H$ NMR spectra, which were assigned using N.O.E. experiments, in which irradiation of the methoxy group of each ester caused specific enhancement of the 6-proton. Merck 9385 silica gel was used for flash chromatography. TLC was performed on Whatman MK6F 60Å silica plates. 4-Nitrophthalic acid (80% technical grade) and anhydrous dimethylformamide (DMF) were purchased from Aldrich, Gillingham, Dorset. Commercial 4-nitrophthalic acid was purified using minor modifications of a published procedure.[1] Trimethylsilyl polyphosphate (PPSE)[2] was purchased from Fluka, Gillingham, Dorset. Light petroleum was the fraction boiling at 40-60 °C and when required was dried by standing over sodium wire overnight. Toluene was dried by heating under reflux in a flask fitted with a Dean-Stark trap until no further water separated. 3-Dimethylaminophenol was purified by vacuum distillation, b.p. 112 °C (2 mm Hg). Procedures involving rhodamines were performed under subdued light. Organic extracts were dried over anhydrous sodium sulphate.

*Dimethyl 4-nitrophthalate* 2.- A solution of commercial 4-nitrophthalic acid 1 (50 g, 237 mmol) in methanol (1 l) containing sulphuric acid (28 ml) was heated under reflux for 8 h. The methanol was removed *in vacuo* and the residue was diluted in ether (500 ml), washed with water (3 x 250 ml) and saturated aq. sodium bicarbonate (4 x 200 ml), dried and concentrated *in vacuo* to give the ester 2 as a yellow solid (36 g, 80%). A sample crystallized from ether as pale needles, m.p. 69-71 °C (lit.[3] 65-66 °C); $\delta_H$

(90 MHz) 3.96 (6 H, s, $CO_2Me$), 7.84 (1 H, d, $J_{5,6}$ 8, 6-H), 8.39 (1 H, dd, $J_{3,5}$ 2, 5-H) and 8.89 (1 H, d, 3-H).

*4-Nitrophthalic acid* **3**.- A suspension of dimethyl 4-nitrophthalate **2** (26.9 g, 112 mmol) in aq. NaOH (2.75 M, 45 ml) was heated under reflux for 1 h, cooled and acidified to below pH 2 with conc. nitric acid. The resulting suspension was extracted with ether (2 x 100 ml; previously washed with 1 M aq. NaOH to remove any ethanol) and the combined extracts were dried. The extract was allowed to evaporate to dryness at atmospheric pressure overnight to give the acid **3** as a white solid (22.4 g, 100%). A sample crystallized from ethyl acetate-light petroleum as pink needles m.p. 164-165 °C (lit.[3] 165 °C); $\delta_H$ (90 MHz, $(CD_3)_2CO$-$CDCl_3$ 1:1) 7.30 (2 H, br s, $CO_2H$), 7.91 (1 H, d, $J_{5,6}$ 8, 6-H), 8.47 (1 H, dd, $J_{3,5}$ 2, 5-H) and 8.66 (1 H, d, 3-H).

*4-Nitrophthalic anhydride* **4**.- A suspension of 4-nitrophthalic acid **3** (40.53 g, 192 mmol) in acetic anhydride (34.5 ml, 366 mmol) was heated for 1 h at 60 °C then under reflux for 10 min. The mixture was allowed to cool, then ground in a mortar with dry light petroleum (2 x 100 ml) to give the anhydride **4** which was filtered and dried under vacuum (33.2 g, 90%). Sublimation of a sample (50 °C, 2 mm Hg) gave clear needles m.p. 122-123 °C (lit.[3] 119 °C); $\delta_H$ (90 MHz) 8.24 (1 H, d, $J_{5,6}$ 8, 6-H), 8.79 (1 H, dd, $J_{3,5}$ 2, 5-H) and 8.83 (1 H, d, 3-H).

*2-[4'-(Dimethylamino)-2'-hydroxybenzoyl]-4- and 5-nitrobenzoic acids* **5** & **6**.- A solution of 4-nitrophthalic anhydride (28.95 g, 150 mmol) and redistilled 3-dimethylaminophenol (21.25 g, 155 mmol) in dry toluene (500 ml) was heated under reflux for 6 h. The toluene was removed *in vacuo* and the residue was dissolved in chloroform (500 ml). The solution was washed with dilute aq. HCl (6 x 200 ml) and water (200 ml), then extracted with saturated aq. sodium bicarbonate (6 x 200 ml). The combined aqueous extracts were acidified to below pH 2 with sulphuric acid and extracted with ether (3 x 200 ml). The combined ether extracts were washed with

water (3 x 200 ml) and extracted with saturated aq. sodium bicarbonate (3 x 200 ml). The combined aqueous extracts were acidified to below pH 2 and extracted with ether (3 x 200 ml). The combined ether extracts were washed with water (200 ml), dried and the solvent was removed *in vacuo* to afford the mixed nitrobenzoic acids **5** and **6** as an orange solid (26.1 g, 53%).

*Methyl 2-[4'-(dimethylamino)-2'-hydroxybenzoyl]-4- and 5-nitrobenzoates* **7** & **8**.-The crude mixed nitrobenzoic acids **5** and **6** (26.1 g, 79 mmol) were dissolved in methanol (500 ml) containing sulphuric acid (14 ml) and heated under reflux for 8 h. The methanol was removed *in vacuo* and the residue was dissolved in chloroform (500 ml), washed with water (300 ml) and saturated aq. sodium bicarbonate (3 x 300 ml), dried and concentrated *in vacuo* to afford an orange solid (26.2 g, 96%). The solid was then crystallized from methanol (1.5 l) to give a yellow-orange solid (15 g). Three further recrystallisations from methanol gave the *4-nitro ester* **7** as yellow needles (3.9 g, 14%), m.p. 177-178 °C; (Found: C, 59.3; H, 4.5; N, 8.3. $C_{17}H_{16}N_2O_6$ requires C, 59.3; H, 4.7; N, 8.1%); $\lambda_{max}$ (EtOH)/nm 259.5 and 351 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 14,100 and 30,300); $\lambda_{max}$ (EtOH-OH$^-$)/nm 346 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 20,700); $\delta_H$ (400 MHz) 3.07 (6 H, s, NMe), 3.78 (3 H, s, $CO_2Me$), 6.12 (1 H, dd, $J_{5',6'}$ 9 and $J_{3',5'}$ 2.5, 5'-H), 6.19 (1 H, d, 3'-H), 6.85 (1 H, d, 6'-H), 8.20 (1 H, d, $J_{5,6}$ 8.4, 6-H), 8.25 (1 H, d, $J_{3,5}$ 2.2, 3-H), 8.36 (1 H, dd, 5-H) and 12.26 (1 H, s, ArOH).

The mother liquor from the first recrystallisation was concentrated by distillation to two-thirds of it's volume, rapidly cooled on ice and the resulting precipitate was collected. This process was repeated until the proportion of isomer **7** in the mother liquor was less than 25% (quantified by integrating the methoxy $^1$H NMR signals corresponding to each isomer). The mother liquor was then evaporated to dryness and the resulting orange solid was recrystallised four times from ethanol to give the *5-nitro ester* **8** as orange prisms (750 mg, 3%), m.p. 164-165 °C (Found: C, 59.2; H, 4.5; N, 8.15. $C_{17}H_{16}N_2O_6$ requires C, 59.3; H, 4.7; N, 8.1%); $\lambda_{max}$ (EtOH)/nm 256.5 and 347 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 8,100 and 17,500); $\lambda_{max}$ (EtOH-OH$^-$)/nm 342 ($\varepsilon$

/$dm^3$ $mol^{-1}$ $cm^{-1}$ 20,100); $\delta_H$ (400 MHz) 3.07 (6 H, s, NMe), 3.81 (3 H, s, $CO_2Me$), 6.09 (1 H, dd, $J_{5',6'}$ 9.1 and $J_{3',5'}$ 2.4, 5'-H), 6.18 (1 H, d, 3'-H), 6.78 (1 H, d, 6'-H), 7.56 (1 H, d, $J_{3,4}$ 8.3, 3-H), 8.46 (1H, dd, $J_{4,6}$ 2.2, 4-H), 8.90 (1 H, d, 6-H) and 12.24 (1 H, s, ArOH).

*2-[4'-(Dimethylamino)-2'-hydroxybenzoyl]-5-acetylaminobenzoic acid* 9.- The ester 8 (1.61 g, 4.68 mmol) was dissolved in acetic acid (450 ml) and 5% Pd-C (300 mg) was added. The mixture was stirred under hydrogen at room temp. and pressure for 5 h, then warmed to 50-60 °C and filtered. The acetic acid was removed *in vacuo* and the residue was suspended in pyridine (25 ml) and acetic anhydride (25 ml, 265 mmol) and stirred for 16 h at room temp. The resulting solution was evaporated to dryness and the residue was dissolved in chloroform (100 ml), washed with 0.2 M aq. HCl (100 ml) and water (100 ml), and concentrated to afford a brown oil. The oil was then dissolved in methanol (30 ml). 10% aqueous NaOH (7.5 ml) was added and the mixture was heated under reflux for 1 h. The methanol was removed *in vacuo*, water (20 ml) was added and the solution was acidified to below pH 2 with 5% aq. sulphuric acid. The resulting precipitate was filtered, washed with water (2 x 20 ml) and dried (50 °C, 2 mm Hg) for 24 h to give the *acetylamino acid* 9 as a yellow solid (1.10 g, 69%). A sample crystallized from methanol as brown prisms m.p. 218-220 °C (dec.) (Found: C, 61.4; H, 5.8; N, 7.6. $C_{18}H_{18}N_2O_5.1/2H_2O$ requires C, 61.5; H, 5.45; N, 8.0%); $\lambda_{max}$ (EtOH)/nm 346.5 ($\varepsilon$/$dm^3$ $mol^{-1}$ $cm^{-1}$ 28,400); $\lambda_{max}$ (EtOH-$OH^-$)/nm 347.5 ($\varepsilon$/$dm^3$ $mol^{-1}$ $cm^{-1}$ 18,900); $\delta_H$ (90 MHz, $d_6$-DMSO-$CDCl_3$ 3:7) 2.13 (3 H, s, MeCO), 3.04 (6 H, s, NMe), 6.06 (1 H, dd, $J_{5',6'}$ 9.5 and $J_{3',5'}$ 2.5, 5'-H), 6.11 (1 H, d, 3'-H), 6.95 (1 H, d, 6'-H), 7.17 (1 H, d, $J_{3,4}$ 8.5, 3-H), 7.94 (1 H, dd, $J_{4,6}$ 2, 4-H) and 8.15 (1 H, d, 6-H).

*2-[4'-(Dimethylamino)-2'-hydroxybenzoyl]-4-acetylaminobenzoic acid* 10.-The ester 7 (2.14 g, 6.26 mmol) was reduced, acylated and saponified, in an identical manner to the ester 7, to give the *acetylamino acid* 10 as a pale yellow solid (1.74 g, 81%). A

sample crystallized from methanol gave brown plates, m p. 228-230 °C (dec.) (Found: C, 62.8; H, 5.1, N, 8.0. $C_{18}H_{18}N_2O_5$ requires C, 63 1; H, 5.3; N, 8.2%); $\lambda_{max}$ (EtOH)/nm 257 and 344 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 18,000 and 28,900); $\lambda_{max}$ (EtOH-$OH^-$)/nm 344 ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$ 19,700); $\delta_H$ (400 MHz, $d_6$-DMSO-$CDCl_3$ 3:7) 2.13 (3 H, s, MeCO), 3.04 (6 H, s, NMe), 6.09 (2 H, m, 5'-H and 3'-H), 6.92 (1 H, d, $J_{5',6'}$ 9.5, 6'-H), 7.69 (1 H, d, $J_{3,5}$ 2.5, 3-H), 7.78 (1 H, d, $J_{5,6}$ 8.5, 6-H) and 7.96 (1 H, dd, 5-H).

*5-Chloroacetylamino-3',6'-bis-(dimethylamino)-spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one* **13**.- A solution of the acid 9 (960 mg, 2.8 mmol), redistilled 3-dimethylaminophenol (800 mg, 5.8 mmol) and PPSE (5 g) in dry DMF (100 ml) was heated under nitrogen at 130 °C for 4 h. The reaction mixture was concentrated *in vacuo* to 10 ml, diluted in 1 M aq. NaOH (200 ml), stirred vigorously for 5 min then extracted with chloroform (3 x 150 ml). The combined extracts were washed with 1 M aq. NaOH (2 x 100 ml), concentrated *in vacuo*, dissolved in conc. HCl-acetic acid (1:1, 200 ml) and heated under reflux for 1 h under nitrogen. The reaction mixture was evaporated to dryness *in vacuo*, diluted in water (100 ml) and again evaporated to dryness. The residual solid was then dissolved in 2 M aq. HCl (200 ml), washed with chloroform (80 ml), basified to above pH 11 with pellets of NaOH and extracted with chloroform (4 x 100 ml). The combined extracts were then washed with 1 M aq. NaOH (2 x 100 ml), dried and concentrated *in vacuo* to afford the crude 5-amino compound 11 as a purple gum (900 mg, 81 %). A portion (720 mg, 1.82 mmol) was dissolved in dry DMF (50 ml), chloroacetyl chloride (145 µl, 1.83 mmol) was added and the mixture was heated under nitrogen at 75 °C for 3 h. The reaction mixture was concentrated, diluted in methanol-chloroform (1:1, 100 ml), mixed with silica gel (2 g) and the solvent was removed *in vacuo*. The silica gel containing the adsorbed compound was added to the top of a flash chromatography column (250 ml silica gel) which was then successively eluted with chloroform (500 ml), methanol-chloroform (1:19, v/v; 250 ml), methanol-chloroform (1:9; 250 ml) and methanol-chloroform (1:4;

750 ml) The major fraction was further purified by flash chromatography (180 ml silica gel), successively eluted with chloroform (500 ml), methanol-chloroform (1:9; 250 ml) and methanol-chloroform (1:4; 750 ml) to afford the *chloroacetamide* **13** as a purple solid (700 mg, 53%) (Found: $M^+$, 478. $C_{26}H_{24}ClN_3O_4$ + H requires *M*, 478); $\delta_H$ (400 MHz, d7-DMF-$CDCl_3$ 3:7) 3.34 (12 H, s, NMe), 4.11 (2 H, s, $ClCH_2CO$), 6.84 (2 H, d, $J_{meta'}$ 2.7, 4'- and 5'-H), 6.97 (2 H, dd, $J_{ortho'}$ 9.4, 2'- and 7'-H), 7.20 (2 H, d, 1'- and 8'-H), 7.22 (1 H, d, $J_{6,7}$ 8.2, 7-H), 8.26 (1 H, dd, $J_{4,6}$ 2, 6-H), 8.77 (1 H, d, 4-H) and 11.40 (1 H, s, HNCO).

*6-Chloroacetylamino-3',6'-bis-(dimethylamino)-spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one* **14**.- The acid **10** (1.167 g, 3.4 mmol), when treated in an identical manner to the acid **9**, afforded first the 6-amino compound **12** and by subsequent reaction as for isomer **13**, the *chloroacetamide* **14** as a purple solid (310 mg, 23%) (Found: $M^+$, 478. $C_{26}H_{24}ClN_3O_4$ +H requires *M*, 478); $\delta_H$ (400 MHz, d7-DMF-$CDCl_3$ 3:7) 3.15 (12 H, s, NMe), 4.25 (2 H, s, $ClCH_2CO$), 6.63 (2 H, d, $J_{meta'}$ 2.3, 4'- and 5'-H), 6.67 (2 H, dd, $J_{ortho'}$ 9, 2'- and 7'-H), 6.89 (2 H, d, 1'- and 8'-H), 7.80 (1 H, d, $J_{5,7}$ 1.9, 7-H), 7.92 (1 H, dd, $J_{4,5}$ 8.5, 5-H), 8.05 (1 H, d, 4-H) and 10.96 (1 H, s, HNCO).

*5-Iodoacetylamino-3',6'-bis-(dimethylamino)-spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one* **15**.- A solution of sodium iodide (0.56 g, 3.75 mmol) in methanol (5 ml) was deoxygenated by bubbling briefly with nitrogen, then added to the 5-chloroacetamide **13** (50 mg, 104.5 μmol). The solution was kept under nitrogen at room temp. for 72 h, then diluted with chloroform (250 ml) and washed with 5% aq. sodium thiosulphate (250 ml) and water (2 x 250 ml). The chloroform solution was diluted with methanol (250 ml), dried and concentrated *in vacuo* to give the *iodoacetamide* **15** as a purple solid (36 mg, 64%); $R_f$ 0.30 ($CHCl_3$-MeOH, 4:1, v/v, developed twice) (Found: $M^+$, 570. $C_{26}H_{24}IN_3O_4$ + H requires *M*, 570); $\delta_H$ (400 MHz, d7-DMF-$CDCl_3$ 3:7) 3.20 (12 H, s, NMe), 4.00 (2 H, s, $ICH_2CO$), 6.69 (2 H,

d, $J_{meta'}$ 2.4, 4'- and 5'-H), 6.75 (2 H, dd, $J_{ortho'}$ 9.2, 2'-H and 7'-H), 6.97 (2 H, d, 1'- and 8'-H), 7.18 (1 H, d, $J_{6,7}$ 8.3, 7-H), 8.09 (1 H, dd, $J_{4,6}$ 2, 6-H), 8.47 (1 H, d, 4-H) and 10.80 (1 H, s, HNCO).

*6-Iodoacetylamino-3',6'-bis-(dimethylamino)spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one* **16**.- The chloroacetamide **14** (50 mg, 104.5 μmol) was treated in an identical manner to its isomer **13** to give the *iodoacetamide* **16** as a purple solid (40 mg, 71%); $R_f$ 0.22 ($CHCl_3$-MeOH, 4:1, v/v, developed twice) (Found: $M^+$, 570. $C_{26}H_{24}IN_3O_4$ + H requires *M*, 570); $\delta_H$ (400 MHz, d7-DMF-$CDCl_3$ 3:7) 3.30 (12 H, s, NMe), 4.03 (2 H, s, $ICH_2CO$), 6.81 (2 H, d, $J_{meta'}$ 2.4, 4'- and 5'-H), 6.93 (2 H, dd, $J_{ortho'}$ 9.4, 2'- and 7'-H), 7.13 (2 H, d, 1'- and 8'-H), 7.89 (1 H, d, $J_{5,7}$ 2, 7-H), 7.96 (1 H, dd, $J_{4,5}$ 8.7, 5-H), 8.21 (1 H, d, 4-H) and 10.87 (1 H, s, HNCO).

*6-Amino-3',6'-bis-(dimethylamino)spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one* **12**.- A solution of the acid **10** (1.2 g, 3.5 mmol), redistilled 3-dimethylaminophenol (959 mg, 7 mmol) and PPSE (5 g) in dry DMF (100 ml) was heated under nitrogen at 130 °C for 4 h. The reaction mixture was concentrated *in vacuo* to 10 ml, diluted in 1 M aq. NaOH (200 ml), stirred vigorously for 5 min and extracted with chloroform (3 x 150 ml). The combined extracts were washed with 1 M aq. NaOH (2 x 100 ml), dried and then concentrated. The resulting solid was then diluted in methanol-chloroform (1:1, 100 ml) and mixed with silica-gel (4 ml) and the solvent was removed under reduced pressure. The silica gel containing the absorbed compound was added to the top of a flash chromatography column (250 ml silica) and successively eluted with chloroform (500 ml), methanol-chloroform (250 ml, 1:19, v/v), methanol-chloroform (250 ml, 1:9) and methanol-chloroform (750 ml, 1:4). The major fraction was then purified again by flash chromatography (180 ml silica), successively eluted with 500 ml chloroform, 250 ml methanol-chloroform (1:9) and 750 ml methanol-chloroform (1:4) to afford the 6-acetamide as a purple solid (400 mg, 26%) (Found: $M^+$, 444. $C_{26}H_{25}N_3O_4$ + H requires *M*, 444); $\delta_H$ (400 MHz, d7-DMF-$CDCl_3$ 3:7) 2.10 (3 H,

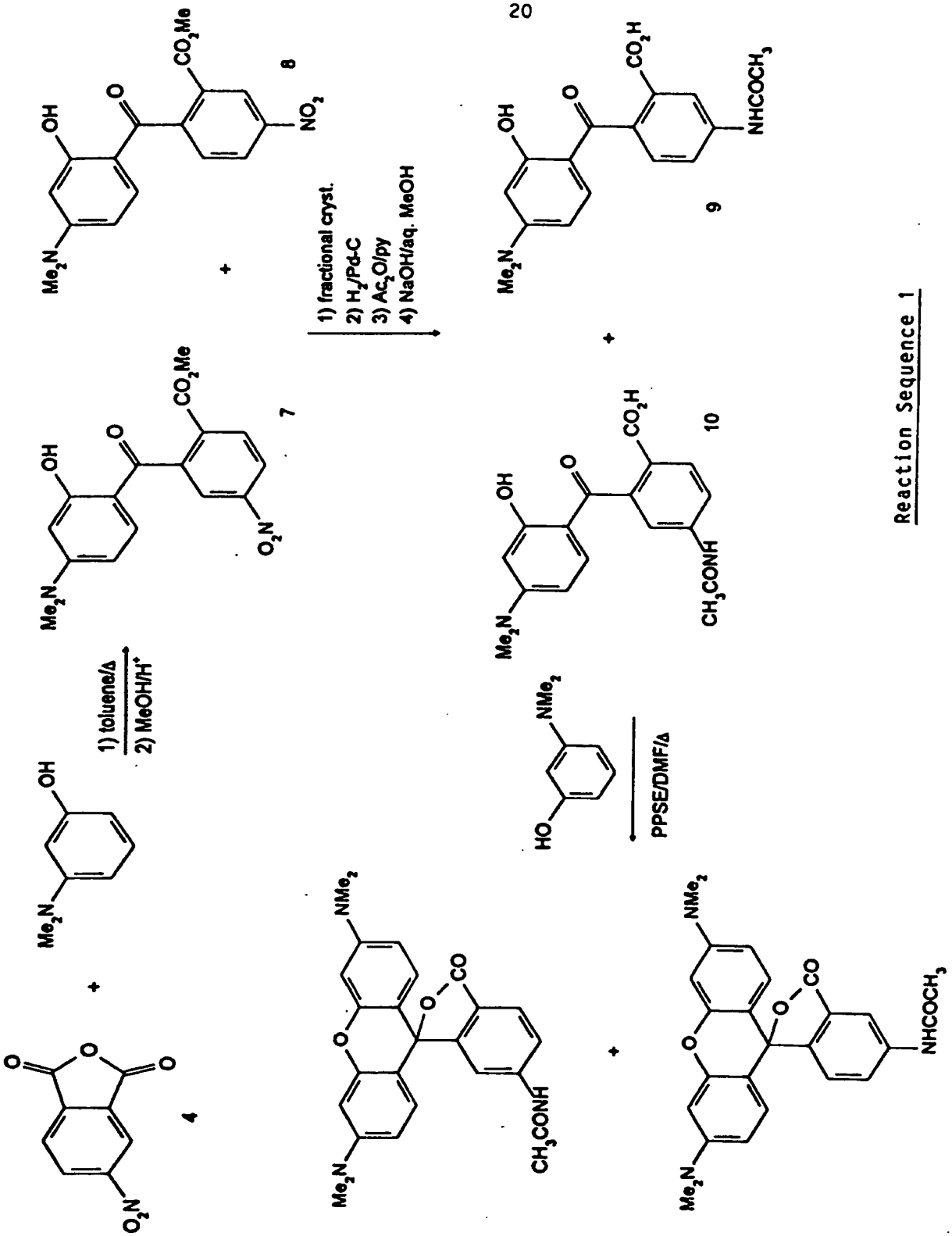
s, $COCH_3$), 3.04 (12 H, s, NMe), 6.50 (2 H, dd, $J_{ortho'}$ 8.5, $J_{meta'}$ 2.7, 2'-H and 7'-H), 6.52 (2 H, d, 4'-H and 5'-H), 6.72 (2 H, d, 1'-H and 8'-H), 7.67 (1 H, d, $J_{5,7}$ 1.5, 7-H), 7.77 (1 H, dd, $J_{4,5}$ 8.9, 5-H), 7.90 (1 H, d, 4-H) and 10.40 (1 H, s, HNCO). A solution of the purified 6-acetamide (200 mg, 450 μmol) in ethanol (100 ml) and 1 M aq. HCl (100 ml) was heated under reflux for 2 h under nitrogen. The reaction mixture was allowed to cool, then diluted with 2 M aq. NaOH (200 ml) and extracted with chloroform (2 x 100 ml). The combined extracts were washed 1 M aq. NaOH (2 x 100 ml), dried and concentrated to afford the *amine* **12** as a purple solid (140 mg, 77%) (Found: $M^+$, 402. $C_{24}H_{23}N_3O_3$ + H requires *M*, 402); $\delta_H$ (400 MHz, $d_7$-DMF-$CDCl_3$ 3:7) 3.04 (12 H, s, NMe), 6.28 (1 H, d, $J_{5,7}$ 1.7, 7-H), 6.51 (2 H, d, $J_{meta'}$ 2, 4'-H and 5'-H), 6.52 (2 H, dd, $J_{ortho'}$ 9.7, 2'-H and 7'-H), 6.79 (2 H, d, 1'-H and 8'-H), 6.83 (1 H, dd, $J_{4,5}$ 8.2, 5-H) and 7.68 (1 H, d, 4-H).

**References**

1 H. Huntress, E. L. Shloss and P. Ehrlich, *Org. Synth. Coll. Vol.*, 1943, **2**, 457; A. I. Vogel, *A Textbook of Practical Organic Chemistry*, Longmans, London, 1956, 3rd ed., p. 967.

2 T. Imamoto, T. Matsumoto, H. Yokoyama, M. Yokohama and K. Yamaguchi, *J. Org. Chem.*, 1984, **49**, 1105.

3 J. Buckingham, ed., *Dictionary of Organic Compounds*, Chapman and Hall, New York, 1982, 5th edn., p. 4226.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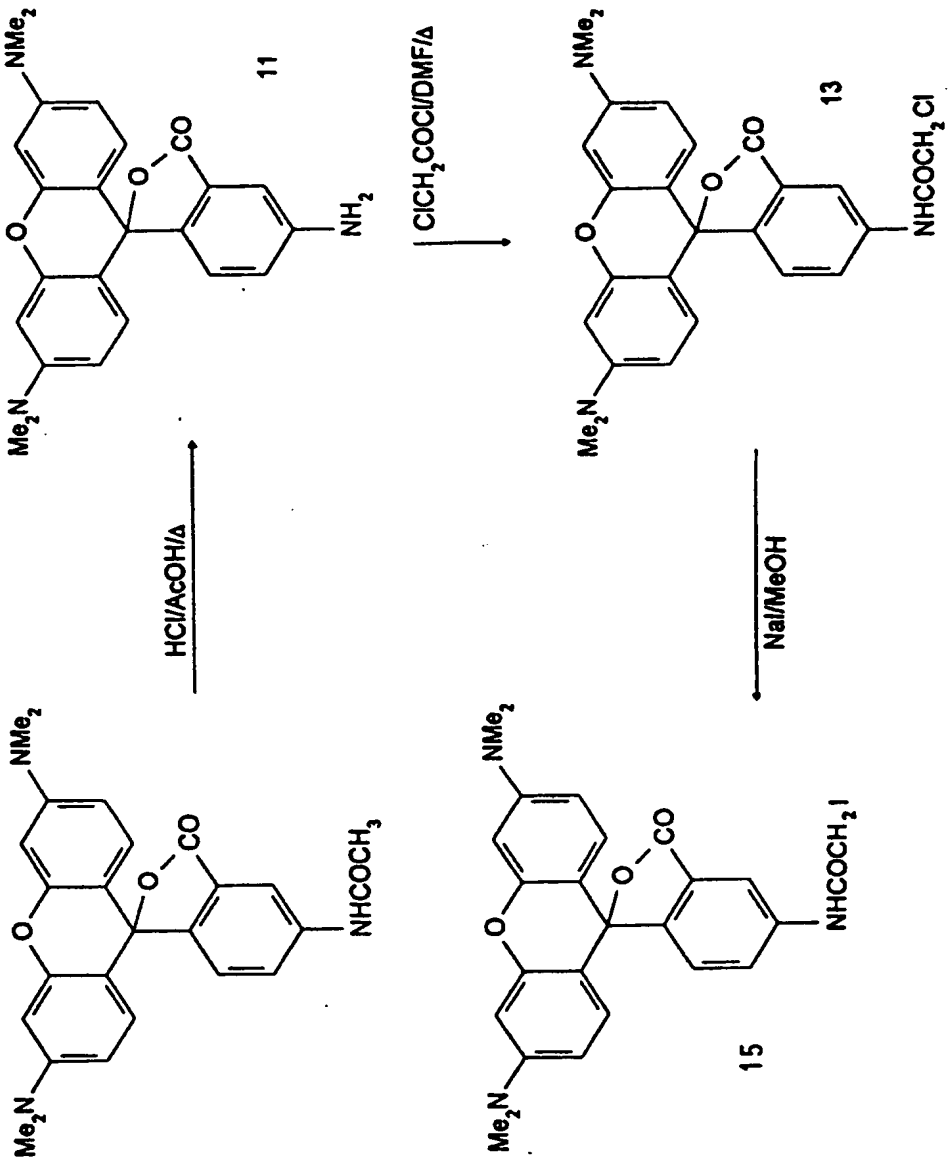
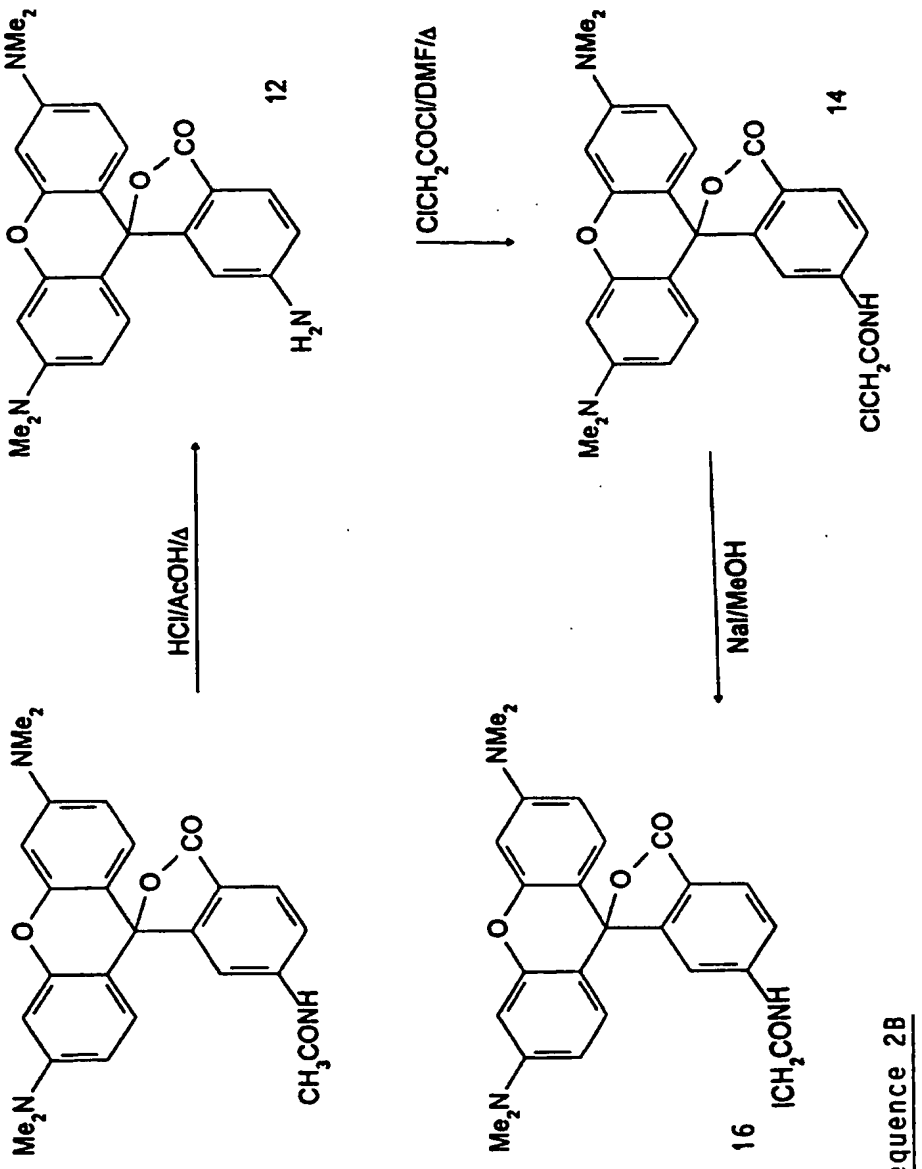
1) fractional cryst.
2) H2/Pd-C
3) Ac2O/py
4) NaOH/aq. MeOH
PPSE/DMF/Δ
Reaction Sequence 1



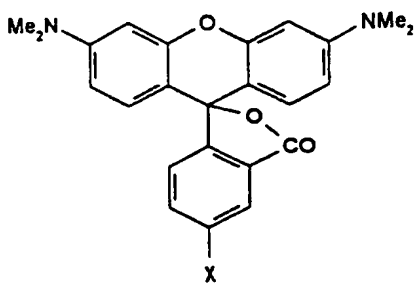

Reaction Sequence 2A




Reaction Sequence 2B

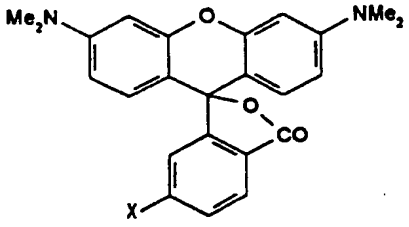
## CLAIMS

1. A method for preparing compounds of the formulae:-




(5 isomer)

or



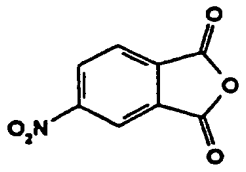

(6 isomer)

or a mixture of such compounds,

where X is bromo, chloro- or iodo- acetamido, maleimido, or amino;

which comprises the following sequence of reactions:-

I)

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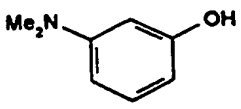
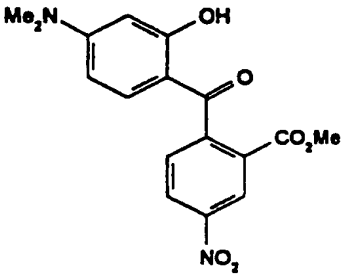
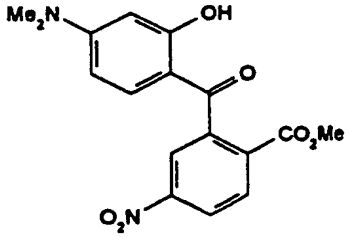



1) toluene/Δ
2) MeOH/H+



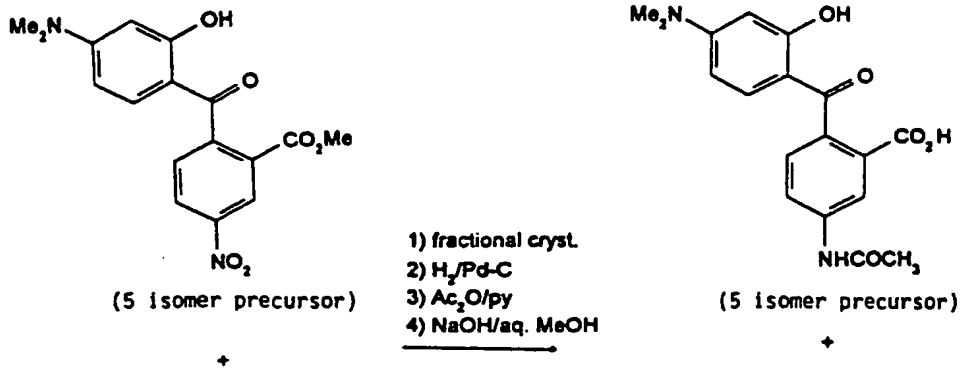

(5 isomer precursor)

+

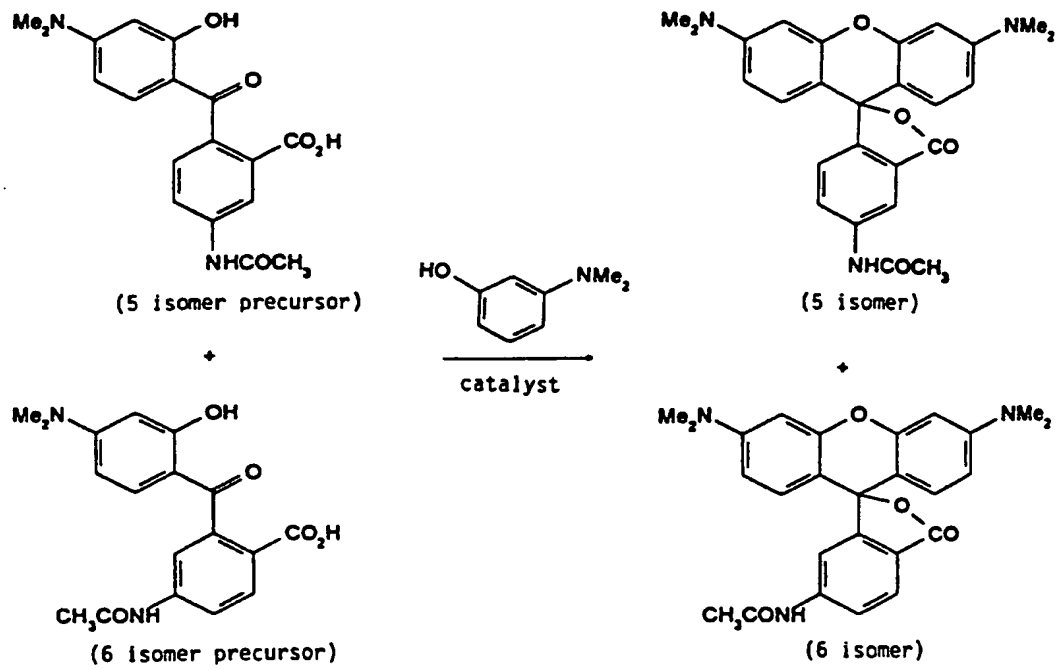



(6 isomer precursor)

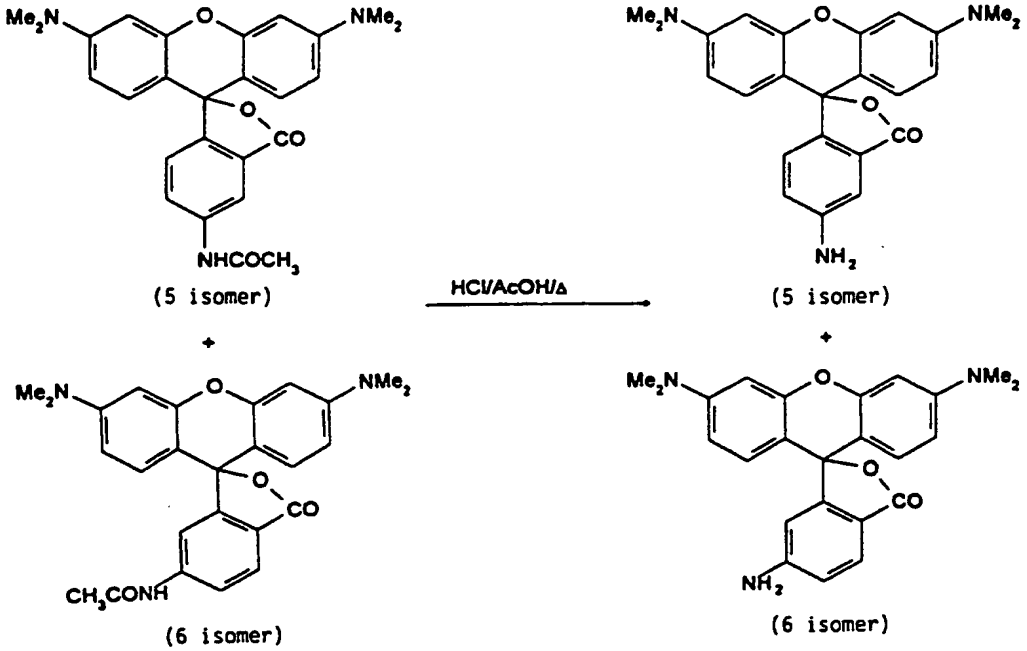
II) reduction of the nitro group:




III) formation of the rhodamine structure in the presence of a catalyst:

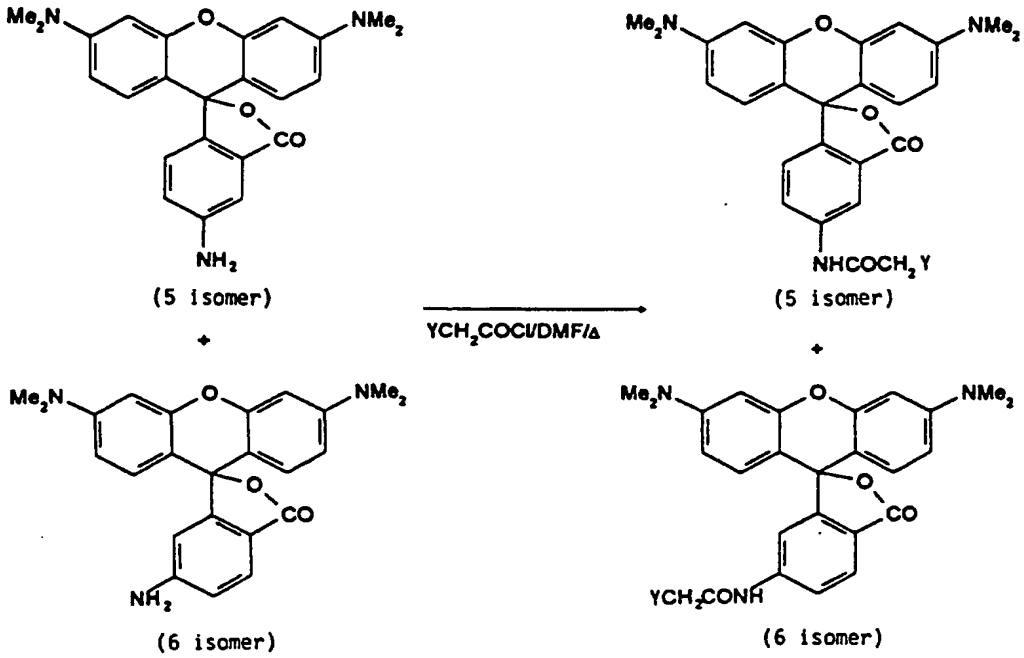


IV) formation of the amine derivative:




and then, optionally,

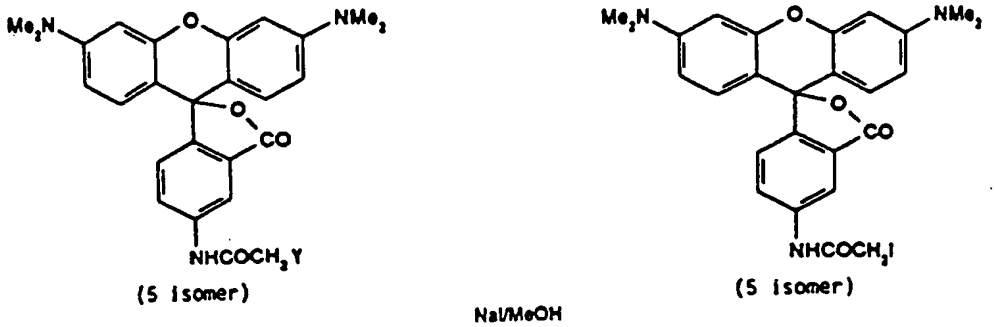
V) conversion of the amine derivative obtained in step IV) to the bromo- or chloro- acetamide derivative:




where Y is Br or Cl;

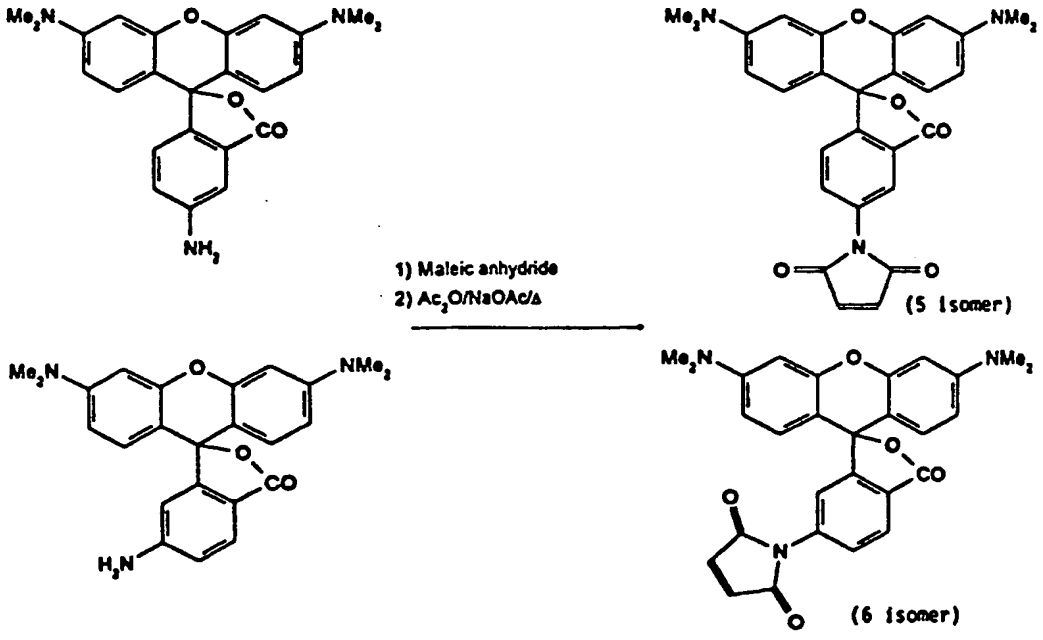
and then, optionally,

VI) conversion of the bromo- or chloro- acetamide derivative obtained in step V) to the iodoacetamide derivative:




or, alternatively,

VII) conversion of the amine derivative obtained in step IV) to the maleimide derivative:



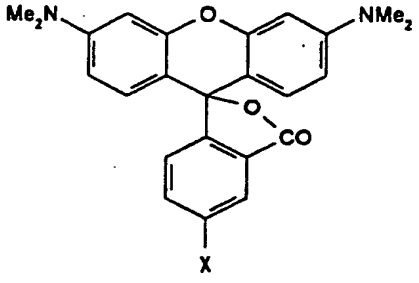
2. A method as claimed in claim 1, wherein the 5 isomer and the 6 isomer precursors are separated from one another at the end of step I) or step II) and before the subsequent steps are performed.

3. A method as claimed in claim 2, wherein the isomer precursors are separated by crystallisation procedures.

4. A method as claimed in claims 1 to 3, wherein X is iodoacetamido.

5. A method as claimed in claims 1 to 4, wherein the catalyst used in step III comprises trimethylsilyl polyphosphate.

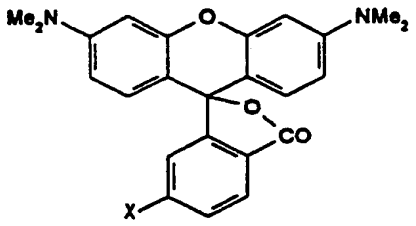
6. A compound of the formula:-




(5 isomer)

where X is as defined above,
and which is in a substantially pure form.

7. A compound of the formula:-




(6 isomer)

where X is as defined above,
and which is in a substantially pure form.

8. A compound as claimed in claims 6 or claim 7,

wherein X is iodoacetamido.

9. A method of investigating or determining protein orientation, structure or movement and which comprises the use of a compound prepared by the method of claims 1 to 5 or a compound as claimed in claims 6 to 8.

# INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/GB 94/02073

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07D493/10 C07D311/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | BIOCHEMISTRY, vol.31, 1992, EASTON, PA US pages 12431 - 12440 K. AJTAI ET AL. 'Stereospecific reaction of muscle fiber proteins with the 5- or 6-isomer of (iodoacetamido)tetramethyl-rhodamine' cited in the application see page 12431 --- | 6-9 |
| X | EP,A,0 297 763 (THEODOROPULOS) 4 January 1989 see example 7 --- | 6 |
| A | US,A,2 937 186 (J.H. BURCKHALTER ET AL.) 17 May 1960 see column 4 --- -/-- | 1,6-9 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 December 1994 | - 4. 01. 95 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+ 31-70) 340-3016 | De Jong, B |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | CHEMICAL ABSTRACTS, vol. 96, no. 13, 29 March 1982, Columbus, Ohio, US; abstract no. 104029q, N.A. RODIONOVA ET AL. 'Tetramethylrhodamine isothiocyanate' page 687 ; see abstract & KHIM. PROM-ST., SER.: REAKT. OSOBO CHIST. VESHCHESTVA, vol.3, 1981 pages 31 - 32 | 6,7 |

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ...nal Application No
PCT/GB 94/02073

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| EP-A-0297763 | 04-01-89 | US-A- | 4822878 | 18-04-89 |
| | | AU-A- | 1846588 | 05-01-89 |
| | | JP-A- | 1070486 | 15-03-89 |
| | | US-A- | 4906749 | 06-03-90 |
| US-A-2937186 | | NONE | | |